DELTA-6 DESATURASE ACTIVITY AND GENE EXPRESSION, TISSUE FATTY ACID PROFILE AND GLUCOSE TURNOVER RATE IN HEREDITARY HYPERTRIGLYCERIDEMIC RATS

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Objective. We have shown previously that the impaired insulin action in hereditary hypertriglyceridemic (hHTg) rat is accompanied by a specific fatty acid (FA) profile in the insulin target tissues, possibly due to a desaturation defect. Thus, the aim of this study was to measure the enzymatic activity and gene expression of Δ-6 desaturase in liver of hHTg rats and the tissue FA composition in relation to insulin action.

Methods. Glucose, triglycerides and insulin in plasma were measured using commercially available enzymatic sets. The hepatic Δ-6 desaturase activity in hHTg rats was determined radiometrically in a microsomal fraction using the 1-^{14}C-linoleic acid as substrate. Δ-6 Desaturase gene expression was measured by the Northern blot technique using a specific cDNA probe. Tissue FA profile was determined by gas chromatography in the total lipid fraction extracted to chloroform. The glucose turnover rate was measured in conscious freely moving animals with the aid of euglycemic hyperinsulinic clamp method.

Results. Tissue triglycerides showed a high accumulation in skeletal muscle of hHTg rats. In the liver of these animals, a defect in Δ-6 desaturase enzymatic activity was found, while the gene expression for Δ-6 desaturase was not changed. Such decreased Δ-6 desaturase activity in the liver was linked to a decrease of Δ-6 desaturase index as calculated from the liver FA composition. Also the concentration of arachidonic acid (a final metabolite in the biosynthesis of polyunsaturated fatty acids of the n-6 series) was significantly decreased in hHTg rat liver. These changes in FA metabolism were accompanied by a decreased glucose infusion rate (a measure of *in vivo* insulin action) required to maintain euglycemia at hyperinsulinemia in hHTg rats, and correlated with the hepatic Δ-6 desaturase activity**.**

Conclusions. 1. hHTg rats showed a reduced activity of the Δ-6 desaturase in liver without any changes in gene expression for this enzyme; 2. such impairment is accompanied by a lower Δ-6 desaturase index (18:2n-6/18:3n-6) found in the liver of these animals and by specific FA profiles in the tissues, particularly regarding the amount of long-chain PUFAs and 18:2n-6 metabolites; and (4) these alterations seem to be related to the impaired insulin action of hHTg rats.

Key words: hHTg rat – Δ-6 desaturase (DS) activity – DS gene expression – Tissue fatty acid spectrum – Insulin action

It has been demonstrated both in rats (Storlien et al. 1991; Wilkes et al. 1998) and humans (Vessby et al. 1994; Vessby 2000) that insulin resistance, one component of the metabolic abnormalities known as the "Syndrom X" (Reaven 1988), is related to a specific fatty acid (FA) pattern in the target tissues of insulin action. Since skeletal muscle is the main determinant of insulin sensitivity, several studies have

shown that FA composition of the phospholipids of the skeletal muscle cell membrane is closely related to insulin sensitivity (Storlien et al. 1996; Storlien et al. 1997; Clore et al. 2000; Park et al. 2000). An increased proportion of saturated fatty acids (SFA), mainly palmitic acid (16:0), and reduced level of long-chain polyunsaturated fatty acids (PUFA) seems to play a critical role in insulin resistance (Baur et al. 1998; Lovejoy 1999; Manco et al. 2000; Mann 2000; Montell et al. 2001). The altered FA profile could be possibly linked to linoleic acid (18:2n-6) metabolic pathways, especially at the level of delta-5 and delta-6 desaturase enzymes (Horrobin 1993; Shin et al. 1995; Vessby 2000).

The hereditary hypertriglyceridemic (hHTg) rat (Vrana and Kazdova 1990) is a nonobese animal model of hypertriglyceridemia (Klimes et al. 1995, Sebokova et al. 1996) and insulin resistance (Stolba et al. 1993; Sebokova et al. 1996; Gasperikova et al. 1997). The impaired insulin action in these animals is paralleled by an abnormal lipid metabolism including changes in FA metabolism (Bohov et al. 1997). However, a question remains whether the specific tissue FA profile of hHTg rats could not be affected by a defect of delta-6 desaturase either at the level of enzymatic activity and/or gene expression. Thus, the aim of this study was to measure the enzymatic activity of and gene expression for the Δ-6 desaturase in liver of hHTg rats, their tissue FA composition and the *in vivo* insulin action in order to prove their relationship, if existing.

Materials and Methods

Materials. [1-^{14}C] Linoleic acid (1.9610 GBq/mmol, 53 mCi/mmol), with more than 98 % radiochemical purity, was purchased from Lacomed SK (Bratislava, Slovak Republic) and was used without further purification. Unlabelled linoleic acid, fatty acids methyl ester standards and others biochemical products were obtained from Sigma Aldrich (Germany). RNAzol™ B (Tel-test, Friendswood, TX, USA) was used for total RNA isolation. RT-PCR kit, Gene Amp, (Perkin Elmer, Norwalk, CT, USA) and [a-^{32}P]-dCTP, (3000 Ci/mmol), (Amersham, Buckinghamshire, UK) allowed us to analyze tissue mRNA expression by the RT-PCR technique. All other chemicals and solvents were of reagent grade from common commercial sources.

Animal study. All experiments reported here were approved by the Institute of Experimental Endocrinology Animal House Ethics Committee. Adult male Wistar rats (245-270 g) were obtained from Charles River Laboratories (Germany). The hereditary hypertriglyceridemic (hHTg) rats (280-340 g) were taken from the colony at this Institute. All animals were housed by two in wire-mesh cages in a temperature-controlled room (22±2 °C) on a 12 hr-light-dark cycle. Rats were fed *ad libitum* commercially available basal diet (Velaz, Prague, Czech Republic) containing protein:lipid:carbohydrate of 26:13:61 cal %, originating from wheat bran (50 wt %), oat bran (10 wt %), fish flour (8 wt %), meat flour (16 wt %), wheat flour (4.5 wt %), soya grit (8 wt %), dry yeast (3 wt %), vitamins and biofactors (0.5 wt %). Five rats from the different strains were sacrificed by decapitation in fed state. Liver and skeletal muscle (mixed *quadriceps femoris*) were removed immediately thereafter, rapidly frozen in liquid nitrogen and stored at -70 °C for later analyses.

Euglycemic hyperinsulinemic clamp. A second set of four rats from each strain was anesthetized by injection of xylazine hydrochloride (10 mg/kg) plus ketamine hydrochloride (75 mg/kg), and fitted with chronic carotid artery and jugular vein cannula (Koopmans et al. 1992). Three days later after 16 hours of overnight fasting, conscious and freely moving animals in metabolic cages were subjected to a 90-min euglycemic hyperinsulinemic clamp (Kreagen et al. 1985; Klimes et al. 1998). Briefly, a continuous infusion of porcine insulin (Actrapid, Novo Nordisk, Denmark) was given at a dose of 6.4 mU/kg /min to achieve plasma insulin concentration in the mid upper physiological range. The arterial blood glucose concentration was clamped at the basal fasting level using a variable rate of glucose infusion. Blood samples were obtained for glucose and insulin determination in all clamp studies at 15 minutes intervals.

Biochemical analyses. Plasma glucose concentration was measured with aid of a Beckman Glucose Analyzer (Fullerton, CA, USA) and insulin with rat RIA kit from Linco (USA). Triglyceride levels in plasma or in tissue lipid extracts were measured by a spectrophotometric method using a specific, commercially available enzymatic set (DOT Diagnostics, Prague, Czech Republic).

Lipids from liver and skeletal muscle were extracted using a slightly modified method of Blight and Dyer (1959) with chloroform – methanol – water (1:1:0.9) mixture. The lipid chloroform extracts were evaporated under a nitrogen stream and immediately dissolved in isopropanol. Triglycerides were examined in the samples after 10 minutes incubation in a shaking water bath at 37 °C using the reagent from the kit. The absorbance was measured at 505 nm with the spectrophotometer Ultrospec 1000 (Pharmacia Biotech, Cambridge, England). For calibration the triolein standard (C 18:1-[cis]-9, Sigma Aldrich, Germany) was used.

Δ-6 Desaturase enzymatic activity. Activity of the hepatic Δ-6 desaturase was determined in microsomes isolated by differential centrifugation. 0.7g of liver was homogenized in 3.5 ml of homogenization buffer containing 10 mM Hepes, pH 7.4, 0.25 M sucrose and 1 mM EDTA. After 10-min centrifugation of the homogenate at 800 x g, the supernatant was centrifuged at 13 000 x g for 20 min. The resulting supernatant was spun at 96 000 x g for 60 min. The microsomal pellets were resuspended in a stock buffer containing 10 mM Hepes, pH 7.4, 0.25 M sucrose and 0.15 M KCl for measurements of the Δ-6 desaturase activity (Garg et al. 1989).

The enzymatic activity was determined in 1 ml assay mixture that contained the following: 10 mmol/l Hepes (pH 7.4), 0.15 mol/l KCl, 0.25 mol/l sucrose, 4 mmol/l ATP, 0.5 mmol/l coenzyme A, 1.25 mmol/l NADH, 0.5 mmol/l niacinamid, 5 mmol/l $MgCl_2$, 62.5 mmol/l NaF, 1.5 mmol/l gluthatione and 100 nmol of [1-14C] linoleic acid. The reaction was started by addition of 1-3 mg of microsomal protein, and the assay mixture was incubated for 10 min at 37 °C. The reaction rate was linear during the period. After stopping the reaction by 2.7 M KOH in methanol, total lipids were saponified by heating the methanolic KOH mixture for 60 minutes at 80 °C. The mixture was than acidified with 8 M HCl (pH 1-2) and fatty acids were extracted with hexane in a 2 step extraction. The fatty acids were then converted to methyl ester by heating up to 100 °C for 30-min with 14 % (w/w) BF_3-methanol, and extracted with hexane. The distribution of radioactivity between the 18:2n-6 substrate and the 18:3n-6 product of Δ-6 desaturase activity was determined by thin-layer chromatography (TLC) with silica gel 60 plates (Merck, 10 x 20 cm, No 1.015626, Darmstadt, Germany) impregnated with 5 % (w/w) $AgNO_3$. Plates were developed in chloroform - ethanol (50:0.375, v/v) for the separation of diens from triens. The spots were made visible under UV light by spraying with 2′, 7′-dichlorofluorescein (0.2 % in methanol), and scraped off directly into the scintillation vials. The radioactivity of the spots was measured in 4 ml of scintillation fluid (Bray) using a liquid-scintillation counter (Beckman). Enzyme activity was expressed as pmol of g-linolenic acid formed from linoleic acid per min per mg of microsomal proteins.

Δ-6 desaturase mRNA. Total liver RNA was extracted using the guanidium thiocyanate-phenol procedure of Chromczynski and Sacchi (1987). (RNAzol™ B, Tel-test, Inc., Friendswood, TX, USA). The quantity and purity of RNA was determined by spectrophotometric measurements at 260, 280 and 230 nm. The relative abundance of delta-6 desaturase was determined by the Northern blot technique. Briefly, the RNA was size fractionated by agarose gel electrophoresis and subsequently transferred to a Nitro Pure membrane (HYBOND C-extra, Amersham, UK, 0.45 mm pore size) by electroblotting. The RNA was fixed to the membrane by vacuum baking (2 hours, 80°C), and the abundance of each transcript of interest was determined by hybridization with the specific cDNA probe. Probes were radiolabelled by using the PRIME-IT RmT kit (Stratagene, La Jolla, CA, USA) and [a-^{32}P]-dCTP (Amersham, Buckinghamshire, UK). Prehybridization and hybridization were performed using the Qick-Hyb hybridization solution (Stratagene, La Jolla, CA, USA). Then the membrane was exposed to X-ray sensitive film (KODAK X-OMAT, Sigma, St. Louis, USA), and the resulting autoradiographic signal was quantified by densitometry using a LCD camera (Eastman KODAK Company, Rochester, NY, USA).

Lipid analyses. Lipids from the rat liver and skeletal muscle were extracted with chloroform-methanol-water (1:1:0.9) (Blight and Dyer 1959) and saponified by heating the methanolic KOH mixture for 60 minutes at 80 °C. The mixture was than acidified with 8 M HCl (pH 1-2) and fatty acids were extracted with hexane in a 2-step extraction. The fatty acids converted to methyl esters by heating at 100 °C for 30-min with 14 % (w/w) BF_3-methanol were extracted to hexane. Determination of fatty acid methyl esters was performed

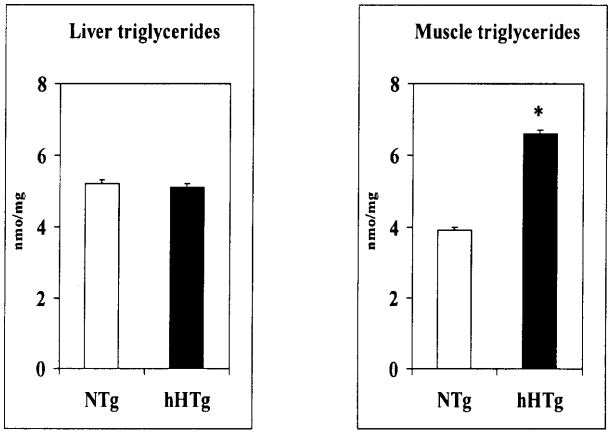

Fig. 1 Triglyceride concentration in liver and skeletal muscle (P<0.05) of hereditary hypertriglyceridemic (hHTg) and control normotriglyceridemic (NTg) rats (mean ± S.E.).

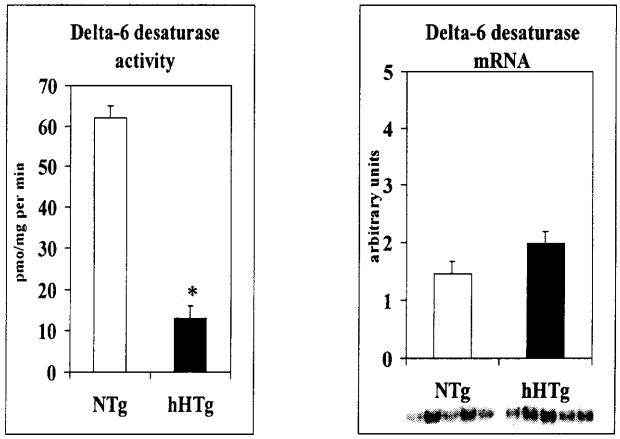

Fig. 2 Delta-6 desaturase activity (P<0.0001) and mRNA in liver of hereditary hypertriglyceridemic (hHTg) and control normotriglyceridemic (NTg) rats (mean ± S.E.).

by gas-liquid chromatography (Finnigan 9001, Austin, Texas) with a flame ionization detection using a SP 2340 fused silica capillary column (Supelco, Bellefonte, Pennsylvania). Data were quantified based on heneicosanoic acid (C 21:0) as an internal standard with the aid of the CSW 1.7 chromatography station (Data Apex, Prague, Czech Republic).

Statistical evaluation. Results were expressed as mean±S.E. Differences between strains were analyzed using an unpaired two tailed t test at the overall significance threshold of P=0.05. Values denoted with a symbol are significantly different.

Results

Animal characteristics (Table 1). The decapitated body weight of hereditary hypertriglyceridemic (hHTg) rats was statistically lower than that of the age matched controls (normotriglyceridemic rats - NTg). Although the increase of fasting insulinemia in hHTg rats did not differ significantly from that in NTg rats, the fasting glucose and postprandial triglyceride (Tg) level was significantly (P=0.05) increased in hHTg rats. The hHTg rats had raised content of Tg in their skeletal muscles when compared to the controls (Figure 1). No Tg accumulation was however seen in the liver.

Δ-6 desaturase activity and mRNA. In the liver of hHTg rat a striking decrease (P=0.001) of the Δ-6 desaturase enzyme activity was found. Nevertheless, gene expression for this enzyme was not changed at all (Figure 2).

Fatty acid profile. Major fatty acids present in total fatty acid fractions of liver and skeletal muscle are listed in Table 2. Thus, in the liver of hHTg rats the palmitic (16:0), linoleic (18:2n-6) and eicosapentaenoic (20:5n-3) acids were significantly elevated, while the level of dihomo-gamma-linoleic (20:3n-6) acid, arachidonic (20:4n-6), alpha-linoleic (18:3n-3) and docosahexaenoic (22:6n-3) acids was decreased. Although the sum of total fatty acids was not changed, the saturated fatty acids (SFA) and trans-fatty acids (trans-FA) were significantly increased in the hHTg rats. Moreover, the hHTg rat liver contained less polyunsaturated fatty acids (PUFA) of the n-3 series, less metabolites of both the PUFA n-6 and the PUFA n-3 and less long-chain (C20-22) PUFA. The most interesting result was a significant

Table 1
Effect of strain on animal characteristics (data as mean ± S.E.).

Strain	NTg	HHTg
Body weight (g)	389 ± 10	328 ± 15*
Glucose (mmol.l^{-1})	3.2 ± 0.2	4.0 ± 0.1*
Insulin (mU/ml)	21.2 ± 4.7	26.6 ± 1.7
Triglycerides (mmol.l^{-1})	1.5 ± 0.2	2.5 ± 0.4*

* P<0.05 vs. control (NTg) values; corrected for multiple analysis
NTg = control normotriglyceridemic rats, hHTg = hereditary hypertriglyceridemic rats.

Table 2
Fatty acid composition in total fatty acid fraction of liver and skeletal muscle hHTg and control (NTg) rats (data as mean ± S.E.).

Fatty acid	Liver		Skeletal muscle	
(wt %)	NTg	hHTg	NTg	hHTg
Palmitic (16:0)	16.8 ± 0.39	20.8 ± 0.36*	19.3 ± 0.68	20.9 ± 0.46
Stearic (18:0)	17.2 ± 0.67	17.8 ± 0.45	11.8 ± 0.33	12.0 ± 0.37
Oleic (18:1n-9)	12.2 ± 0.66	11.5 ± 0.58	12.4 ± 0.80	12.2 ± 1.53
Linoleic (18:2n-6)	14.6 ± 0.24	17.4 ± 0.17*	17.9 ± 0.19	16.6 ± 0.08*
Gamma-linolenic (18:3n-6)	0.20 ± 0.02	0.16 ± 0.01	0.06 ± 0.06	0.04 ± 0.03*
Dihomo-gamma-linolenic (20:3n-6)	1.15 ± 0.03	0.94 ± 0.05*	1.00 ± 0.06	0.90 ± 0.03
Arachidonic (20:4n-6)	18.7 ± 0.73	14.6 ± 0.45*	9.0 ± 0.34	8.8 ± 0.56
Alpha-linolenic (18:3n-3)	0.53 ± 0.04	0.39 ± 0.04*	0.36 ± 0.02	0.30 ± 0.04
Eicosapentaenoic (20:5n-3)	1.2 ± 0.04	1.7 ± 0.04*	0.71 ± 0.05	0.81 ± 0.04
Docosahexaenoic (22:6n-3)	8.6 ± 0.51	5.8 ± 0.07*	13.9 ± 0.72	13.5 ± 0.92
Total FA (g/100g)	4.8 ± 0.35	4.9 ± 0.14	1.04 ± 0.03	1.10 ± 0.06
SFA	35.1 ± 0.44	39.9 ± 0.29*	32.6 ± 0.39	34.9 ± 0.29*
MUFA	15.4 ± 0.72	14.5 ± 0.63	17.2 ± 1.06	16.9 ± 1.64
PUFA n-6	35.2 ± 0.97	33.5 ± 0.44	29.0 ± 0.63	26.8 ± 0.67
PUFA n-3	12.0 ± 0.63	9.6 ± 0.07*	18.8 ± 0.85	18.9 ± 1.11
Trans FA	2.0 ± 0.07	2.4 ± 0.06*	2.4 ± 0.10	2.4 ± 0.08
PUFA n-6M	20.6 ± 0.73	16.1 ± 0.48*	11.0 ± 0.45	10.3 ± 0.60
PUFA n-3M	11.5 ± 0.60	9.2 ± 0.06*	18.4 ± 0.85	18.6 ± 1.15
n-6/n-3	3.0 ± 0.26	3.5 ± 0.07	1.5 ± 0.06	1.4 ± 0.05
C20-22 PUFA	31.9 ± 0.17	25.1 ± 0.49*	29.4 ± 2.42	28.8 ± 1.74
18:3/18:2*1000	13.9 ± 1.69	8.9 ± 0.47*	3.3 ± 0.29	2.6 ± 0.16
20:4/20:3	16.3 ± 0.18	15.7 ± 0.69	9.1 ± 0.27	9.7 ± 0.50
n-6M/18:2n-6	1.4 ± 0.03	0.9 ± 0.03*	0.61 ± 0.02	0.62 ± 0.03
n-3M/18:3n-3*10	21.7 ± 1.09	24.6 ± 2.83	51.1 ± 3.34	67.8 ± 11.08

* P<0.05 vs. control (NTg) values; corrected for multiple analysis
SFA = saturated fatty acids, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids

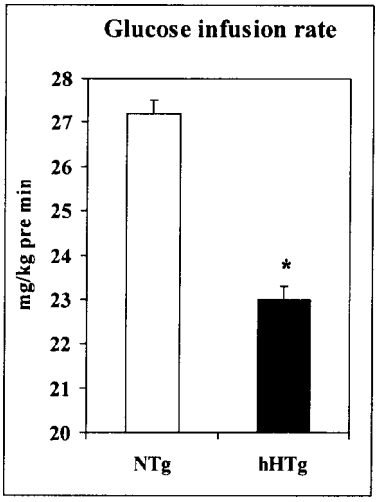

Fig. 3 Results of euglycemic hyperinsulinemic clamp: effect of strain on glucose infusion rate (mean ± S.E.) (P<0.05). NTg = control normotriglyceridemic Wistar rats, hHTg = hereditary hypertriglyceridemic rats.

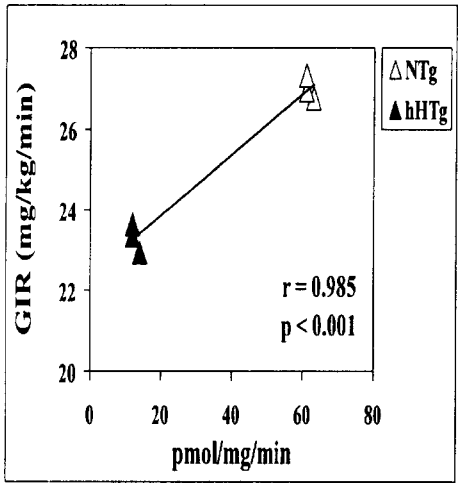

Fig. 4 Correlation analysis between glucose infusion rate (GIR) and delta-6 desaturase activity. NTg = control normotriglyceridemic rats , hHTg = hereditary hypertriglyceridemic rats.

decrease of the delta-6 desaturase index (18:3n-6/18:2n-6), and of the PUFA n-6 metabolites ratio to linoleic acid (n-6M/18:2n-6) in liver of hHTg rats (Table 2) that underlined an impairment of delta-6 desaturase enzyme activity we found in liver of these animals.

The profile of skeletal muscle fatty acids did not differ so much between the strains. A decrease in the linoleic and gamma-linolenic acid levels was seen in the hHTg rats. On the other hand, the saturated fatty acids were elevated in skeletal muscle of hHTg rats when compared to control NTg rats (Table 2).

The results of euglycemic hyperinsulinemic clamp study. The glucose infusion rate (GIR, an index of insulin sensitivity) was significantly decreased in hHTg rats when compared to control. Thus, the hHTg rat are insulin resistant (Figure 3). It is interesting that the GIR strongly correlated with the liver delta-6 desaturase activity (Figure 4).

Discussion

Long chain polyunsaturated fatty acids such as arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) play a pivotal role in a number of biological functions (Uauy et al. 1992, Neuringer et al. 1994, Birch et al. 1998, Cho et al. 1998). Clarke et al. (1997) showed that such 20 and 22-carbon PUFAs are vital components of membrane phospholipids and govern the expression of a wide array of genes including those encoding proteins involved with lipid metabolism, thermogenesis, and cell differentiation. Their availability is greatly dependent upon the rate of desaturation of linoleic acid (18:2n-6) and alpha-linolenic acid (18:3n-3) by delta-6 desaturase (Sprecher 1981). This is a microsomal enzyme with the pronounced activity in the liver (Salway 1994).

Our biochemical and molecular analysis revealed major differences in FA metabolism between the two rat strains investigated. Liver and skeletal muscle were particular interest objects of our study. Liver is known for its high lipid and carbohydrate metabolic rate, while skeletal muscle is the most important tissue for the insulin-stimulated glucose metabolism.

In accordance with our previous findings (Klimes et al. 1994) we showed that hypertriglyceridemia of hHTg rats is accompanied by an increased accumulation of Tg in skeletal muscles. However, the main findings suggests that the *in vivo* insulin resistance of this animal model is accompanied by lower Δ-6-desaturase enzyme activity. Consequently, changes in FA composition in total FA fraction of liver and skeletal muscle occurred. In particular, in the liver lower amounts of 18:2n-6, 20:4n-6, 22:6n-3, PUFA n-6 metabolites and C20-22 PUFA were found. In skeletal muscle, also the gamma-linolenic acid (18:3n-6) content was decreased, which nicely reflects inhibition of the Δ-6 desaturase activity. Interestingly, hepatic Δ-6 desaturase gene expression was not changed in hHTg rats. This is in contrast with the data of Rimoldi et al. (2001), who found a sevenfold decrease in the abundance of the hepatic Δ-6 desaturase mRNA in streptozotocin-induced diabetic rats. Although we do not have a plausible explanation for this discrepancy, it is clear that Rimoldics rats had a lack of insulin. On the contrary, the hHTg rats have rather higher levels of circulating insulin when compared with controls.

Previous studies showed that the activity of D-6 desaturase is reduced in both, the experimental diabetes as well as in humans with Type 1 and Type 2 diabetes mellitus (Horrobin 1988, Mohan and Das 2000). Horrobin (1993) suggested that the high requirement for 18:2n-6 in diabetic animals is attributed to a reduced conversion of 18:2n-6 to 18:3n-6 and further metabolites. Indeed, these further metabolites seem to perform most of the biological functions of the 18:2n-6. Hence, higher levels of this acid are required to allow for adequate levels of the downstream substances to be produced. Other investigators found that the a reduced Δ-6 desaturase activity *in vitro* is usually accompanied by an impairment of the Δ-5 and Δ-4 desaturases (Brenner 1982, Poisson 1985, Poisson and Blond 1985). However, this reduction can be reversed by acute insulin treatment. It is though not yet clear whether this is a direct action of insulin on the enzyme, or whether the effect is mediated by an intermediary molecule.

Most of the studies have been performed in rats made diabetic by streptozotocin or alloxan. In these models, the impaired desaturation is also reflected in fatty acid composition of several tissues including blood, liver, muscle (heart and skeletal) and kidney. The changes in FA pattern are usually indicated by elevated 18:2n-6 and reduced 20:4n-6 levels (Kanzaki et al. 1987, Parinandi et al. 1990, Mimouni et al.

1992). The decreased level of long-chain PUFA n-6 in the adipocyte plasma membranes of streptozotocin-induced diabetic rats has been suggested to be associated with decreased insulin binding in isolated adipocytes. However, several others factor, such as FA elongation and oxidation, and membrane lipid turnover may also contribute to these alterations.

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